# Proteoglycans modulate renal inflammation

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# Chapter 7

## General discussion, Conclusions and Future perspective

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## 7.1 General discussion

In this thesis we aimed to study the contribution of proteoglycans (PG) in innate immune-derived renal injury in transplantation and native kidney diseases. To this end we designed studies to look at the different aspects of innate immunity and their interaction with PGs in proteinuria, renal ischemia/reperfusion (I/R) and transplantation. As discussed in the introduction (Chapter 1) of this thesis, innate immunity as the first line of defense, is involved in the pathogenesis of renal injury in proteinuria (1, 2), I/R (3) and transplantation (4).The innate immune system consists of cellular and humoral components. Leukocytes build the cellular arm, while complement system and cytokines form the humoral arm of innate immune system. Involvement of inflammation and complement system in proteinuria, I/R and transplantation has already been established (1, 3–7). The alternative pathway (AP) of the complement system is spontaneously and constantly activated on biological surfaces and may lead to cell lysis, opsonization and amplification of inflammatory signals. The AP plays an important role in a number of renal diseases (8). Mutations in components of the AP can play a role in the pathophysiology of renal damage by hampering AP regulation. In proteinuric renal disease, proteinuria can activate the AP by two different mechanisms. First, the serum proteins filtered in urine were shown to stimulate renal tubular cells to produce complement factors and second, the complement factors present in the ultrafiltrate can activate the pathway on tubular cells (1). In renal I/R, complement activation occurs mostly via the AP and the Mannose-Lectin binding pathway (9, 10). In renal transplantation, the AP is activated in both acute and chronic rejection (11, 12). Since AP of complement plays an important role in tubule-interstitial injury in all three abovementioned settings, namely proteinuria, I/R and transplantation, in this thesis we mainly focused on the contribution of PGs in AP-mediated tubulo-interstitial injury.

Properdin is the only positive regulator of the AP and it can initiate the AP by binding to cell surfaces (13). However its ligand on cells was not yet identified. Factor H is the main inhibitor of the AP both on the cell surface and the fluid phase. Factor H is known to interact with PGs on the cell surface (14). Moreover, both properdin and factor H are recognition molecules of AP which can differentiate between host cells and pathogens (14). Considering these facts, we were interested to investigate properdin and PGs as well as factor H and PGs interaction in proteinuric rat models. In Chapter 2 we identified tubular heparan sulfate proteoglycans (HSPG) as the ligands for properdin which is present in the ultrafiltrate. Furthermore, we demonstrated that interaction of properdin with tubular HSPG leads to AP activation on renal tubular cells. Additionally, we assessed the molecular binding properties of glycosaminoglycans (GAG) for binding to properdin. In Chapter 3 we confirmed previous studies showing factor H-PG interaction on tubular cells. Besides we showed the ability of factor H to inhibit AP activation on tubular cells via interaction with tubular HSPGs. Based on our results, we concluded that during proteinuria both factor H and properdin are present in the ultrafiltrate, and both can interact with tubular HSPGs (Fig. 7.1).

Next, we compared the binding properties of GAGs for both properdin and factor H. A series of competition ELISA assays were designed to determine whether or not properdin and factor H compete for the same motif on HS or they recognize different binding sites. This is important from the therapeutical point of view, to design a drug

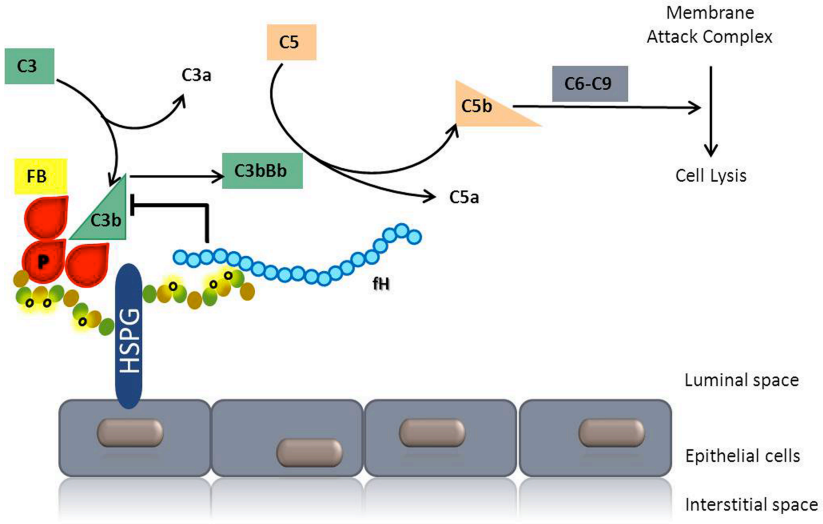


**Figure 7.1: Schematic picture summarizing the findings in Chapter 2 and 3.** It is proposed that factor H (fH) and properdin (P), present in ultrafiltrate during proteinuria, bind to tubular heparan sulfate proteoglycans (HSPGs). Binding of properdin to tubular HSPGs leads to alternative pathway activation on tubular cells while factor H interaction with tubular HSPGs prevents the activation of alternative pathway.

which targets the properdin interaction with PGs, however does not influence the factor H function. The ideal drug to suppress the activation of AP during proteinuria, will inhibit properdin from initiating the AP while it does not interfere with factor H-tubular HS interaction. In Chapter 3 we demonstrated that properdin and factor H did not compete with each other to interact with HSPGs. Thus, they bind to different motifs on tubular HSPGs (as illustrated in Fig. 7.2). It seemed that properdin interacts easier with HSPGs while factor H needs higher sulfation in order to bind to HSPGs. We thus showed that these interactions could be manipulated by certain low anticoagulant heparinoids in favor of AP inhibition. Due to a high degree of sulfation, unfractionated heparin inhibited both properdin and factor H interaction with HSPGs. Besides, heparin, as mentioned before, has anticoagulant activity which is not desirable in physiologic context. Our results demonstrated the ability of some low anticoagulant heparinoid preparations in selective blockade of properdin-HSPG interaction while factor H-HSPG interaction remains intact in presence of these heparinoids. These data provide a basis for targeted, specific heparinoid interventions in proteinuric renal diseases.

It is known that the AP is activated after renal transplantation, and it has also been shown that AP plays a role in both acute and chronic rejection (6, 12). As the above data support a role for therapeutic effects of specific heparinoids, we hypothesized that heparionoid intervention might provide renoprotection in chronic transplant dysfunction. To test this hypothesis, in Chapter 4 we used a rodent transplantation model to intervene with heparin and two different non-anticoagulant heparinoids with different levels of sul-

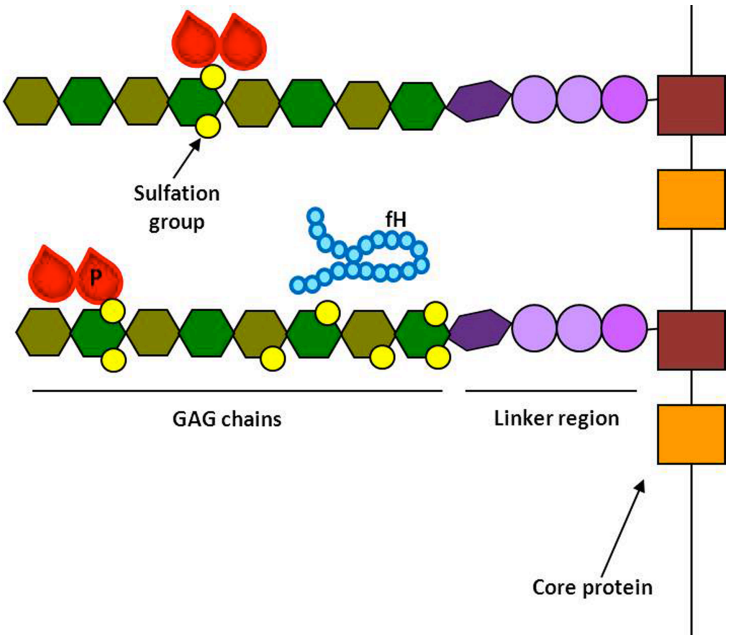


**Figure 7.2: Picture illustrating the binding motifs on the glycosaminoglycan (GAG) chains of heparan sulfate proteoglycans.** Factor H (fH) and properdin (P) both bind to heparan sulfate proteoglycans. However they interact with different motifs on HSPG. Modifications (e.g. sulfation groups) on GAG chains define the binding. Properdin is able to bind to a lower sulfated GAG chain, while factor H needs a higher degree of sulfation for binding. Therefore, it indicates the importance of tubular HSPG pattern in regulation of AP in proteinuria.

fation. Over time we followed loss of renal function by measuring serum urea, creatinine and the development of proteinuria. We also quantified leukocyte influx and expression of complement factors in the transplanted kidneys. Renal function loss was similar for heparin(oid) treated and vehicle treated animals. However, the R-O heparin treated group showed a tendency towards reduction of proteinuria. We found a reduced number of CD45+ inflammatory cells in the tubulo-interstitium of R-O heparin treated animals but simultaneously the same group showed significantly increased properdin expression, mainly in the glomeruli. Factor H expression was similar in heparin(oid) treated and vehicle treated animals. However, we could not show any difference in activation of the AP (measured by C3 and MAC deposition in renal tissue). Since properdin freshly secreted from neutrophils is monomeric and does not bind to HSPG (unpublished data, Zaferani et al), we suggest that the higher expression of properdin in R-O heparin treated group is not properly functional to activate the AP. Nevertheless, it would be interesting to look for neutrophil influx in these animals in tubulo-interstitium and glomeruli.

So, the results of intervention were modest, and mainly limited to a lower influx of CD45 positive cells. These data support the concept that heparinoids can modify processes of renal damage, but also, that the effect is not straightforward. Our data also support the concept that specificity of heparinoids is important for the possible therapeutic effect.

Hence, heparinoids should be chosen carefully to target the desired interaction. Moreover, pharmacokinetic considerations, such as dose-response, deserve proper attention. It has been shown that exogenous heparinoids can interfere with the growth factor signaling and inflammation (15–17). As we discussed earlier in this thesis, PGs are involved in several levels of innate inflammatory response. Leukocyte rolling, adhesion, activation, extravasation and migration are partly mediated by PGs. In addition, binding of PGs to various chemokines, cytokines and complement factors has an important role in innate immune system activation. Therefore, heparinoids can potentially be used to target any of these interactions. Further research is needed to characterize the binding properties of PGs for selectins, integrins, chemokines, cytokines and complement factors in order to enable us to target the desired interaction more efficiently. In addition to (low molecular weight) heparins, other heparinoids with low anticoagulant activity such as K5 preparations (18), other natural GAGs (e.g. some heparan sulfates or chondroitin sulfates or GAGs from algae or invertebrate animals), or synthetic HS mimetics could be considered for potential therapeutic use.

Basement membrane PGs have been shown to be involved in leukocyte recruitment to the injured tissue (19). Two of the basement membrane PGs are collagen XV and collagen XVIII. These two collagens/PGs are best known for their anti-angiogenic activity via their C-terminal domains. Chapter 5 focuses on cellular components of innate immunity and on the role of basement membrane PGs in leukocyte recruitment to renal tissue upon I/R. We used knockout mice for basement membrane proteoglycans, namely collagen XV, collagen XVIII and their compound mutant to evaluate the influx of neutrophils and macrophages into renal tissue after I/R injury. Indeed, the compound mutant mice lacking both basement membrane HSPGs (collagen XV and XVIII) showed a decrease in number of neutrophils and macrophages to kidney comparing to wildtype mice. Moreover, these double mutant mice showed less tubular damage and reduced tubular cell activation. Serum urea levels showed a reduction in double mutant mice which is indicative of a better renal function after I/R. Less tubular damage and a better renal function might be due to a milder inflammatory response after I/R in collagen XV and XVIII deficient mice. However, the exact mechanism of the collagen XV and XVIII involvement in inflammatory cell influx is not well understood. Taken together our data with the results from previous studies, three possible mechanisms can be suggested. First, broadened (vascular) basement membranes (20) in the KO mice can be a durable barrier for leukocyte influx. Second, collagen XV and XVIII like other basement membrane PGs might interact with leukocyte adhesion molecules and facilitate leukocyte migration (19, 21, 22). Third, basement membrane collagen XV and XVIII can play a role in chemokine gradient formation guiding leukocyte migration. We concluded that basement membrane HSPGs play an important role in leukocyte recruitment and renal tissue damage after I/R. Our results thus showed a new role for collagen XV and collagen XVIII as HSPGs besides their anti-angiogenic function. HSPGs have been shown to interact with complement factors (Chapter 1). Moreover, complement system has an established role in pathogenesis of I/R injury. Thus, it would be interesting to check for the complement activation in this model as well. Future research can unravel a potential role of basement membrane collagen/HSPGs in development of complement derived I/R injury. Innate immune system activation is notable in renal fibrosis. Involvement of Toll-like receptors, macrophages and dendritic cells in renal fibrosis has been shown (23, 24). Depletion of macrophages has been

shown to be beneficial and ameliorates renal fibrosis after various injuries, while adoptive transfer of macrophages aggravates the fibrotic lesions, demonstrating their profibrotic roles in renal fibrogenesis (25). As we showed in this chapter, PGs play an important role in leukocyte influx and activation after injury in kidney. Thus, it would be interesting to dissect this area more, since it can bring a new approach to target progression of renal fibrosis.

In Chapter 6, we hypothesized that factor H polymorphisms can influence the graft outcome after renal transplantation. In a large transplant cohort database, we checked for the association of both donor and recipients factor H tagSNPs with end stage renal disease, graft loss and acute rejection in renal transplant recipients. Our results confirmed the involvement of donor factor H gene variants in acute rejection after transplantation. The associations were found in donor SNPs and not in recipients SNPs, which is suggestive of the importance of intra-renal production of factor H. Moreover, we showed two of the factor H SNPs to be a risk factor for end stage renal disease in a case-control analysis. All together the results of this chapter indicate the involvement of AP in pathogenesis of both end stage renal disease and acute rejection. However, we must mention that these data. In a single transplant cohort, still require independent confirmation in a different cohort – which would be a transplant cohort for confirmation of association with acute rejection, and an ESRD cohort for the association with ESRD. Other studies have been reported no association between the mannose-lectin binding proteins variants and renal graft outcome after transplantation (26). Moreover, no difference was found in genetic variants of C3 and renal transplantation outcome (27). Taken together, these results suggest AP involvement rather than mannose-lectin pathway or classical pathway in acute renal graft rejection. However, this issue needs further investigation.

## 7.2 Conclusions and Future perspective

In this thesis, we investigated some mechanisms of innate immune system involvement in the pathogenesis of renal damage. We proposed a novel mechanism for proteinuria-derived tubular injury via PGs interaction with complement factors. It would be interesting to expand this research line to other complement factors specially the AP components and terminal complement components in proteinuria. Although our results showed a defined role for tubular HSPG in proteinuria derived renal injury, *in vivo* studies would be essential to confirm the clinical use of heparinoids in reduction of tubule-interstitial inflammation secondary to proteinuria. Rodent models of chronic proteinuria like adriamycin induced nephropathy might be a good animal model to be treated with heparinoids. However, the binding properties of the heparinoids for various complement factors and inhibition of the various complement pathways should be checked *in vitro* beforehand.

We also showed a novel role for basement membrane PGs in I/R induced renal injury. Our model, however, was a relatively mild I/R model (25min bilateral occlusion) which does not lead to extensive post-ischemic fibrosis. For investigating the role of PGs in renal fibrosis, it would be better to choose a more severely fibrotic model like unilateral ureteric obstruction in which we can investigate the role of PGs in the fibrotic process regarding their interaction with innate immune components. Based on the results, a therapeutic approach with some heparinoids can be considered.

Furthermore, we were able to show the role of genetic variation in the AP in occurrence of acute rejection and progressive renal function loss resulting in end stage renal disease in native kidneys. This is the first time that this association has been shown in a transplant cohort analysis, and should be replicated in an independent cohort. Additionally, more mutations in complement factors can be checked in association with renal graft outcome. The possible role of PGs genetic variants in the renal transplant population would be interesting to investigate as well. The genetic association studies provides a better insight to the involvement of complement-PG interaction in the process of renal function loss and serves as a starting point for further mechanistic studies.

Taken together, it is clear that insight in the molecular interactions in renal diseases is important to allow the design of novel strategies for treatment. Immune suppressive drugs used after transplantation mainly target the adaptive immune system, while the activation of innate immune system in the disease process is mostly neglected. For achieving a better outcome in treatment of renal diseases including transplantation, considering the role of innate immune system could provide novel targets for intervention. Our studies in this thesis provide novel insights in the role of PGs in renal inflammation in relation to innate immune derived injuries. However, further research is required to bring these findings close to application.

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